EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1091

TOXICITIES OF TNT AND RDX TO TERRESTRIAL PLANTS IN FIVE SOILS WITH CONTRASTING CHARACTERISTICS

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July 2013

U.S. ARMY RDECOM

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-07-2013	Final	Apr 2001 – Feb 2007

4. TITLE AND SUBTITLE
Toxicities of TNT and RDX to Terrestrial Plants in Five Soils with Contrasting Characteristics

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Simini, Michael; Checkai, Ronald T.; Kuperman, Roman G.; Phillips, Carlton T.; Kolakowski, Jan E.; and Kurnas, Carl W.

5d. PROJECT NUMBER
SERDP CU-1210

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, ECBC, ATTN: RDCB-DRT-E, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1091

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program
4800 Mark Center Drive, Suite 17D08, Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
SERDP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT-LIMIT 200 WORDS
This study was designed to produce scientifically defensible benchmark data for the development of ecological-based soil screening levels for 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) for terrestrial plants. The physical and chemical properties of different soils may alter the bioavailability and toxicity of TNT and RDX to plant species. We investigated individual ecotoxicities of TNT and RDX to alfalfa (*Medicago sativa* L.), Japanese millet (J. millet; *Echinochloa crus-galli* L. [Beauv.]), and perennial ryegrass (*Lolium perenne* L.) in five natural soils that varied in pH, organic matter, and clay contents. On the basis of EC_{50} values (the TNT or RDX concentrations that produce 50% reductions in selected measurement endpoints) and 95% confidence intervals for shoot fresh and dry masses (SDM), TNT weathered-and-aged (W-A) in soils significantly reduced ($p < 0.05$) growth of alfalfa, J. millet, and perennial ryegrass. RDX W-A in soil was moderately inhibitory to growth of J. millet and perennial ryegrass in all five soils but did not affect growth of alfalfa. No statistically significant correlations between TNT toxicity and these soil properties were identified; however, statistically significant ($p < 0.05$) inverse relationships were identified between the RDX EC_{50} values for perennial ryegrass SDM and soil clay content and pH.

15. SUBJECT TERMS

Ecological soil screening level	TNT	Toxicity assessment	Weathering-and-aging
Terrestrial plants	RDX	Natural soil	Bioavailability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	54	19b. TELEPHONE NUMBER *(include area code)* (410) 436-7545

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PREFACE

The work described in this report was authorized under Strategic Environmental Research and Development Program project no. SERDP CU-1210. The work was started in April 2001 and completed in February 2007.

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This report has been approved for public release.

Acknowledgments

The authors thank Drs. Geoffrey I. Sunahara and Jalal Hawari from Biotechnology Research Institute, National Research Council of Canada for their contributions of methodology development, interlaboratory participation in quality control assurance measures for analytical determinations of RDX and TNT, and data exchange during execution of this project.

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TOXICITIES OF TNT AND RDX TO TERRESTRIAL PLANTS IN FIVE SOILS WITH CONTRASTING CHARACTERISTICS

1. INTRODUCTION

Many sites associated with military operations involving munitions manufacturing, disposal, testing, and training have been contaminated with elevated levels of explosives and related materials in soil. Concentrations of explosives in soil have been reported to exceed 87,000 mg kg^{-1} for 2,4,6-trinitrotoluene (TNT) and 3,000 mg kg^{-1} for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Simini et al., 1995). Although these energetic materials (EMs) can be persistent in the environment, their effects on soil biota have not been sufficiently investigated. As a result, scientifically defensible screening values, which could be used in ecological risk assessment (ERA), are not currently available for these explosives in soil. Scientifically based ecological soil screening levels (Eco-SSLs) are needed to identify contaminant explosive levels in soil that do not present a potential ecological concern onsite, and therefore do not need to be considered in baseline ecological risk assessment (BERA). To address this problem, the U.S. Environmental Protection Agency (U.S. EPA), in conjunction with stakeholders, is developing Eco-SSL values for contaminants most frequently found at Superfund sites (U.S. EPA, 2005). Eco-SSLs are defined as the concentrations of chemicals in soil that, when not exceeded, will protect terrestrial ecosystems from unacceptable harmful effects. These Eco-SSL values can be used in a screening-level ERA (SLERA) to identify those contaminants in soil that warrant additional evaluation in a BERA and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. After an extensive literature review (U.S. EPA, 2005), the Eco-SSL workgroup determined that there was insufficient information regarding explosives to support the derivation of Eco-SSL values for terrestrial plants. Our study was designed to fill this knowledge gap.

Published studies on the phytotoxicity of explosives to higher terrestrial plants are scant (Gong et al., 1999; Sunahara et al., 2001; Hannink et al., 2002; Robidoux et al., 2003; Rocheleau et al., 2005, 2006, 2008, 2010, 2011). Winfield et al. (2004) found that exposure to RDX (up to 4000 mg/kg soil) during early life stages resulted in adverse responses in sensitive terrestrial plants such as sunflower and sainfoin. In field studies, corn, tomato, and lettuce died when exposed to 580 mg RDX/kg soil and 1720 mg TNT/kg soil (Price et al., 1997; Pennington and Brannon, 2002). Wild-type tobacco plants exposed to 1 mM nitroglycerin could not germinate normally and exhibited severe stunting of root and shoot development (French et al., 1999). Although a benchmark value of 100 mg RDX/kg soil was determined by Talmage et al. (1999), confidence in the single benchmark is low because the available data are insufficient to derive and establish an Eco-SSL value according to well-defined criteria (U.S. EPA, 2005).

Several terrestrial plant toxicity tests, for which standardized protocols have been developed (ASTM, 2002; Environment Canada, 2005; International Organization for Standardization [ISO], 1995; U.S. EPA, 1996), have been used to assess toxicities and derive protective benchmark values for a variety of chemicals (Sunahara et al., 2001; Stephenson et al., 2002). We adapted procedures from the ASTM and U.S. EPA protocols for the studies reported

herein. Procedures used in the present studies were selected for their ability to measure chemical toxicity to ecologically relevant test species during chronic assays. Explosives in soils at many contaminated sites have been subjected to weathering-and-aging processes for years. Therefore, special consideration was given to assessing the effects of weathering-and-aging on the toxicity of EMs to terrestrial plants for Eco-SSL development. Weathering-and-aging of chemicals in soil may reduce exposure of terrestrial plants to EMs due to photodecomposition, hydrolysis, reactions with organic matter (OM), sorption, precipitation, immobilization, occlusion, microbial transformation, and other fate processes. These processes may result in dramatic reductions in the amounts of chemicals that are bioavailable (Esteve-Núñez et al., 2001; Renoux et al., 2000). Conversely, transformation products produced during the weathering-and-aging process may be more toxic than the parent material to plants and other soil organisms (Rocheleau et al., 2005; Kuperman et al., 2005, 2006; LaChance et al., 2004). Results from later studies with TNT weathered-and-aged (W-A) in Sassafras sandy loam soil showed that plant growth decreased for Japanese millet (J. millet) but increased for alfalfa and perennial rye (Rocheleau et al., 2006). This approach was later applied successfully toward establishing plant benchmarks for 2,4-dintrotoluene (2,4-DNT) (Rocheleau et al., 2010). We incorporated a weathering-and-aging procedure in these tests to more accurately simulate conditions in the field that may affect exposure of terrestrial plants to EMs.

Studies reported herein were designed to produce scientifically defensible benchmark data for the development of Eco-SSL values for TNT and RDX for terrestrial plants in aerobic upland soils that meet specific criteria (U.S. EPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that:

- Experimental designs for laboratory studies were documented and appropriate;
- Both nominal and analytically determined concentrations of chemicals of interest were reported;
- Tests included both negative and positive controls;
- Chronic or life cycle tests were used;
- Appropriate chemical dosing procedures were reported;
- Concentration-response relationships were reported;
- Statistical tests used to calculate the benchmarks and levels of significance were described; and
- The origins of test species were specified and appropriate.

Tests were also conducted in five different field soils having different physicochemical characteristics that may alter the bioavailability of TNT and RDX, including soils that sustain high relative bioavailability of EMs.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization

The soils used in these studies included the following:

- Teller sandy loam (TSL), a fine-loamy, mixed, active, thermic Udic Argiustoll collected from agricultural land of the Oklahoma State University Perkins Experiment Station, Payne County, OK;
- Sassafras sandy loam (SSL), a fine-loamy, siliceous, semiactive, mesic Typic Hapludult collected from an open grassland field in the coastal plain on the property of the U.S. Army Aberdeen Proving Ground, Harford County, MD;
- Kirkland clay loam (KCL), a fine, mixed, superactive, thermic Udertic Paleustoll collected from Payne County, OK;
- Richfield clay loam (RCL), a fine, smectitic, mesic Aridic Argiustoll collected from Texas County, OK; and
- Webster clay loam (WCL), a fine-loamy, mixed, superactive, mesic Typic Endoaquoll collected from Story County, IA.

According to Eco-SSL criteria (U.S. EPA, 2005), the qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered "very high" for TSL and SSL, "medium" for KCL and WCL, and "low" for RCL. During soil collection in the field, vegetation and the organic horizon were removed, and the top 15.2 cm of the A-horizon was collected. Soil was sieved through a 5 mm mesh screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, and stored at room temperature. Soil was then analyzed for physical and chemical characteristics (Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD). Results of these analyses are presented in Table 1.

Table 1. Mean Physical and Chemical Characteristics of Five Field Soils (n = 3)

Soil Property	TSL Soil	SSL Soil	KCL Soil	RCL Soil	WCL Soil
Sand (%)	65 (1.0)	70 (0.7)	37 (0.33)	30 (30.3)	33 (0.6)
Silt (%)	22 (1.0)	13 (0.9)	34 (0.33)	42 (1.7)	39 (0.3)
Clay (%)	13 (0.0)	17 (0.3)	28 (0.33)	28 (0.9)	28 (0.7)
Texture	Sandy loam	Sandy loam	Clay loam	Clay loam	Clay loam
Cation exchange capacity (cmol kg^{-1})	4.3 (0.03)	5.5 (0.1)	10.3 (0.09)	27.6 (1.40)	20.8 (0.1)
Organic matter (%)	1.4 (0.03)	1.3 (0.06)	2.6 (0.06)	3.3 (0.03)	5.3 (0.09)
pH	4.4 (0.03)	5.2 (0.03)	6.4 (0.03)	7.4 (0.06)	5.9 (0.03)
Water-holding capacity (%)	13 (0.6)	18 (4.0)	20 (1.0)	21 (1.5)	23 (0.18)

Notes: Analyses were performed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. Standard errors of the means are shown in parentheses.

2.2 Test Chemicals

The EMs TNT (Chemical Abstracts Service [CAS] no. 118-96-7; purity 99.9%), and RDX (CAS no. 121-82-4; purity 99%) were obtained from the Defence Research Establishment Valcartier of the Canadian Ministry of National Defence (Val Bélair, QC, Canada). Boric acid (H_3BO_3; CAS no. 10043-35-3; purity 99.99%; Alfa Aesar; Ward Hill, MA) was used as the positive control in all tests. High-performance liquid chromatography (HPLC)-grade acetone (CAS no. 67-64-1) was used to prepare the TNT and RDX solutions before the soils were amended. Acetonitrile (ACN; CAS no. 75-05-8; HPLC grade), methanol (CAS no. 67-56-1; chromatography grade; purity 99.9%), and calcium chloride ($CaCl_2$; CAS no. 10043-52-4; reagent grade) were used for soil extractions and analytical HPLC determinations. Certified standards of TNT and RDX (AccuStandard; New Haven, CT) were used in HPLC determinations. ASTM Type I water (18 MΩ cm at 25 °C; ASTM, 2004) was used throughout the toxicity studies. It was obtained using Milli-RO 10 Plus followed by Milli-Q PF Plus systems (Millipore; Bedford, MA). The same grade of water was used throughout the analytical determinations. Glassware was washed with phosphate-free detergent and sequentially rinsed with tap water, ASTM Type II water (>5 MΩ cm at 25 °C), analytical reagent-grade nitric acid 1% (v/v), and ASTM Type I water.

2.3 Soil Amendment Procedures

Studies were performed separately and independently for TNT or RDX in freshly amended (FA) and W-A soil to determine toxicity benchmark values for TNT or RDX in each exposure type. During the soil amendment procedure, TNT or RDX was amended into separate aliquots of soil using an organic solvent (acetone) as a carrier. This was necessary to distribute the TNT or RDX evenly and uniformly to a large soil surface area, which would have been difficult to achieve if solid chemical crystals had been added to soil. Carrier control soils were amended with acetone only. Soil was spread to a thickness of 2.5 cm. The TNT or RDX solution was pipetted evenly across the soil surface, and the volume of solution added at any one time did not exceed 15% (v/w) of the soil dry mass. After the solution was added, the volumetric flask was rinsed twice with a known volume of acetone, which was also pipetted onto the soil. If the total volume of solution required to amend the soil exceeded 15% (v/w), the solution was added in successive stages. Between additions, the acetone was allowed to evaporate for a minimum of 2 h within a darkened chemical hood. Amended soil was air-dried overnight (minimum of 18 h) in a darkened chemical hood to prevent photolysis of the EM. Each soil treatment sample was then transferred into a fluorocarbon-coated, high-density polyethylene container and mixed for 18 h on a three-dimensional rotary mixer.

2.4 Weathering-and-Aging of TNT and RDX in Soil

Standardized methods for weathering-and-aging of explosives in soil are not available. We have developed approaches that simulate, at least in part, the weathering-and-aging processes in soil to more closely approximate the exposure effects on soil biota in the field (Kuperman et al., 2003, 2005; Simini et al., 2003, 2006). Air-dried soil batches were amended with several concentrations of TNT or RDX. In a greenhouse, the dried soil batches were initially hydrated in open glass containers with ASTM Type I water to 60% of the water-holding

capacity (WHC) of each soil. Soil was then subjected to alternating cycles (up to 3 months duration) of hydration and air-drying at ambient temperature in a greenhouse. Each soil treatment was weighed and readjusted to its initial mass by weekly addition of ASTM Type I water. Any soil surface crust that formed during the week was broken with a spatula before water was added. After the conclusion of the EM weathering-and-aging procedures, each soil treatment was brought to 95% of its WHC 24 h before toxicity tests were started.

Soil treatments with TNT concentrations representing low, intermediate, and high levels were monitored periodically during the weathering-and-aging process to determine the time when TNT concentrations were effectively stabilized or had declined to ≤5% of the initial concentration in FA soil treatments with the highest rate of decrease. Nominal TNT concentrations selected for monitoring in these studies were: 20, 100, 200, and 300 mg kg^{-1} in TSL; 50, 100, 200, and 400 mg kg^{-1} in SSL or KCL; 5, 25, 100, and 500 mg kg^{-1} in RCL; and 40, 100, 200, and 400 mg kg^{-1} in WCL. The respective times determined for each TNT–soil pairing were then designated for termination of the weathering-and-aging procedures within treatments for that soil and commencement of the corresponding definitive toxicity tests.

Previous studies have shown that RDX did not significantly degrade under aerobic conditions, and that soil invertebrate toxicities did not significantly change ($p \leq 0.05$) when RDX-amended soils were subjected to the weathering-and-aging process (Simini et al., 2003; Kuperman et al., 2003; Dodard et al., 2005). Therefore, after soils were amended with RDX, concentrations in soils were not monitored until the RDX weathering-and-aging procedures were concluded after 3 months. Immediately before toxicity testing was started, RDX concentrations were analytically determined in each soil.

2.5 Measurement of Soil pH

The pH values of the test soils were determined at the beginning of each definitive toxicity test using a method adapted from the *Soil Survey Laboratory Methods Manual* (USDA, 2004). Five grams of ASTM Type I water was added to 5 g of soil. The soil slurry was vortexed for 10 s every 5 min for 30 min, then 1 min before pH measurement, the soil slurry was vortexed again for 10 s. While the slurry was gently stirred, the soil pH was analytically determined in the solution above the soil surface until the pH reading stabilized. Before measurement of soil pH for each definitive test, the pH electrode was rinsed thoroughly with ASTM Type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed, and blotted. The electrode was also rinsed with ASTM Type I water and blotted before each pH measurement.

2.6 ACN Extraction of TNT or RDX from Soil

At the beginning of each definitive test, each batch of control soils and the RDX- or TNT-treated soils were subsampled in triplicate. ACN was used to extract TNT or RDX from each sample, then EM concentrations were analytically determined in accordance with U.S. EPA Method 8330A (U.S. EPA, 2007). Before extraction, soil subsamples for analytical determination were hydrated to 60% of their respective WHCs for 24 h, in accordance with the procedures in "Weathering-and-Aging of TNT or RDX in Soil" (Section 2.4). The soil dry fraction (dry weight/wet weight) was determined in triplicate from subsamples of each treatment

concentration. For extraction, 2 g soil samples were collected from the soil batch treatments and controls and placed into respective 50 mL polypropylene centrifuge tubes, and 10 mL of ACN was added to each tube. Samples were vortexed with the ACN for 1 min, then sonicated in darkness for 18 h at 20 °C. Five milliliters of each supernatant was transferred into glass tubes that contained 5 mL of $CaCl_2$ solution (5 g/L). The supernatant was then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe cartridge, and 1 mL of each filtered solution was transferred into an HPLC vial. Soil extracts were analyzed, and concentrations were quantified by HPLC.

2.7 Adapted Toxicity Characteristic Leaching Procedure (ATCLP) Extraction of TNT from Soil

During ACN extraction, both the nonaccessible (nondissolved crystalline plus adsorbed) and the water-soluble fractions of TNT or RDX are measured. Consequently, although conservative values are obtained, use of U.S. EPA Method 8330A can result in overestimation of the amount of explosive available to an exposed organism because the bioavailability of an organic compound having an octanol–water partition coefficient (log K_{ow}) of <5 (1.6 for TNT and 0.90 for RDX; Monteil-Rivera et al., 2009) for uptake by a soil organism is primarily determined by the fraction dissolved in the soil interstitial water (Belfroid et al., 1994, 1996; Savard et al., 2010). Therefore, in addition to ACN extraction, the water-soluble fraction of TNT was extracted from soil using an ATCLP (Haley et al., 1993). TNT concentrations determined using this method better estimate the actual field soil–water conditions that exist as a result of respiration by soil biota and are perceived to measure the intensity factor of the bioavailable fraction of chemicals in soil.

At the beginning of each definitive test, in addition to extraction with ACN, TNT was extracted from a subsample of each batch of control soils and TNT-treated soils using the ATCLP method (Haley et al., 1993). The ATCLP is a modification of the toxicity characteristic leaching procedure (TCLP; 40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The procedure was modified by substituting CO_2-saturated water for acetic acid to acidify the water used for extraction and thereby simulate the soil–water conditions that exist as a result of respiration by soil biota and retain the effects of the natural buffering capacity of the soil. The CO_2-saturated water was not recharged once it was added to the soil. All ATCLP extractions were performed in triplicate. For each subsample replicate from the treatment concentration batches for TNT, 4 g of soil were transferred in triplicate into 20 mL vials. Sixteen milliliters of CO_2-saturated water (pH 3.8–4.0) was added to each vial, and the vials were immediately sealed. Each soil sample was vortexed for 45 s before being mixed for 18 h on a rotary (end-over-end) mixer (30 rpm) at room temperature in darkness (40 CFR Part 268.41). The solutions were allowed to settle for at least 2 h, and supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equal volume of ACN was added to each filtered soil extract before HPLC analysis was performed. Herein, TNT concentrations determined using the ATCLP soil extraction procedure are referred to as the EM water-soluble fractions. Nominal and analytically determined concentrations from the definitive tests are shown in Tables 2 through 10.

ATCLP-based extractions were not conducted in studies with RDX because multiple concentrations selected for definitive toxicity tests exceeded the aqueous solubility of RDX (42 mg L^{-1} at 20 °C, Monteil-Rivera et al., 2004).

2.8 Analytical Determinations

Soil extracts were analyzed by reversed-phase HPLC using a modified EPA Method 8330A. The method was modified by adjusting the flow rate of the 50/50 methanol–water mobile phase to 1.0 mL min^{-1} rather than 1.5 mL min^{-1}. A 25 cm × 4.6 mm × 5 µm particle size C-18 column was used for all determinations. For HPLC, Beckman System Gold analytical instrumentation (Beckman Coulter; Brea, CA) was used, which consists of a model 126 programmable solvent module, a model 168 diode array detector, and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard) of each EM in a 50/50 water–ACN solution in a range of concentrations appropriate for each set of determinations. Blanks and standards were placed intermittently between samples. The method detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil. All chemical concentrations in soil were expressed on dry mass basis.

2.9 Phytotoxicity Assessment

Phytotoxicity assessment methods were adapted from two standardized protocols, *Standard Guide for Conducting Terrestrial Plant Toxicity Tests* (ASTM, 2002) and *Ecological Effects Test Guidelines, Early Seedling Growth Toxicity Test* (U.S. EPA, 1996). Plant testing with TNT included range-finding tests to identify the range of concentrations to use in definitive tests, and definitive tests to determine benchmark concentrations in soil that may be used to develop Eco-SSLs. Testing with RDX included range-finding, limit, and definitive tests. Initially, limit tests were performed because on the basis of previous study results (Rocheleau et al., 2005, 2006), it was suspected that no statistically significant effects would occur at the highest soil concentration used in these studies (10,000 mg kg^{-1}). A limit test is a toxicity test in which, if no statistically significant negative effects occur relative to the control at a preselected maximum dose, no further testing is required at greater exposure levels.

For each test, the phytotoxicity test was designated as valid if the number of seedlings that emerged in control treatments was at least 75% of the total number of seeds planted, and if the mean survival rate for the control seedlings was at least 75% at the conclusion of the test. Measurement endpoints used in these studies were:

- The number of emerged seedlings tallied after the emergence incubation period (7 days),
- The number of emerged seedlings tallied at conclusion of the test, and
- The composite fresh- and dry-shoot masses (SFM and SDM, respectively) per replicate treatment (20 plants) at the conclusion of the test.

The test species in these studies were *Medicago sativa* (L.) var. Canada no. 1 (alfalfa), *Echinochloa crus-galli* (L.) P. Beauv. var. Common no. 1 (J. millet, barnyard grass), and *Lolium perenne* (L.) var. Express (perennial ryegrass). *M. sativa* seed stock was obtained from William Dam Seeds (Dundas, Ontario, Canada; catalog no. 550) and was lot-packed and tested in 2000. Alfalfa-clover nitrogen-fixing bacteria for *M. sativa* were obtained from Southern States Cooperative (Richmond, VA; catalog no. 111-08000; lot no. 3092002). *E. crus-galli* seed stock was obtained from Labon (Boucherville, Quebec, Canada; catalog no. 300-380; lot no. 9-6). *L. perenne* seed stock was from Pickseed (St. Hyacinthe, Quebec, Canada; catalog no. 1269) and was supplied by Labon.

Conventional 4 in. planting pots (9.7 cm top diameter, 7.3 cm bottom diameter, 7.0 cm height) were used as plant growth containers. Washed pea gravel (200 g) was placed into the bottom of each pot, and cheesecloth was placed atop the pea gravel to prevent soil loss. Once the TNT and RDX amendments had been W-A in the soil, 200 g of soil (dry-weight basis) per treatment level was placed into each pot. For each plant species experiment, each treatment replicate received 20 seeds that were planted at a soil depth equal to twice the seed diameter. Two and four replicate pots per treatment were used in the range-finding and definitive tests, respectively.

After seeds were sown, the soil was hydrated with ASTM Type I water to 95% WHC. The initial mass of each pot was recorded, and all pots were placed into a plant growth chamber (model PGC-9/2; Percival Scientific; Perry, IA). The following test conditions were maintained in the growth chamber: temperature, 25 ± 3 °C (light) and 20 ± 3 °C (dark); relative humidity (RH), 75 ± 5%; photoperiod, 16 h (light) and 8 h (dark); and light intensity, 200 to 240 µmol s^{-1} m^{-2} radiation (photosynthetically active). Light intensity was measured continuously using a Quantum light sensor (Spectrum Technologies; Plainfield, IL).

Each pot was weighed daily and adjusted back to its initial mass at test commencement by addition of dilute Miracle-Gro solution (Scotts Miracle-Gro Company; Marysville, OH). To avoid the effects of nutrient deficiencies that may exist in natural soils, a dilute solution (288 mg L^{-1}) was prepared that included Miracle-Gro fertilizer (15% total nitrogen [calculated as N], 30% available phosphate [calculated as P_2O_5], 15% soluble potash [calculated as K_2O], 0.02% boron, 0.07% copper [chelated], 0.15% iron [chelated], 0.05% manganese [chelated], 0.0005% molybdenum, 0.06% zinc [chelated], and 1.14% ethylenediaminetetraacetic acid as a chelating agent). The duration of each test was 14 days after emergence of at least 50% of the seedlings in the respective untreated (control) soil. Five to seven days after sowing of the plant species, 50% of the respective seeds had emerged in control treatment pots. Seedling emergence values were then recorded, and each test was continued for another 14 days. At the conclusion of each test (after the 14 day exposure period), the numbers of surviving plants per treatment replicate were recorded for each study. The aerial (shoot) portions of the plants were then harvested, weighed, placed in a drying oven at 65 ± 2 °C for 24–72 h, and reweighed until a constant weight was measured. The shoots were harvested by cutting with a razor blade at the transition point between the above-ground shoot and the below-ground roots. Harvested shoots (fresh or dry) were placed into preweighed envelopes and weighed. Weights were recorded to the nearest 0.001 g.

2.9.1 Phytotoxicity of TNT

To identify the range of concentrations to use in the definitive tests, range-finding tests were performed with each of the three plant species with TNT amended into the five soils. Nominal TNT concentrations used in the range-finding tests were 0 mg kg^{-1} (no amendment) and 10, 100, 200, 400, and 800 mg kg^{-1}. Planting procedures and cultural practices for the range-finding tests were the same as those described in Section 2.9, “Phytotoxicity Assessment”.

Definitive TNT toxicity tests were performed by individually testing each of the three plant species with each of the five soils. Nominal treatment levels for TNT amended into

the five soils, based on the results of the range-finding tests using the three plant species, were 0 mg kg^{-1} (no amendment); 0 mg kg^{-1} (acetone carrier control); and 1, 10, 20, 40, 80, 160, and 320 mg kg^{-1}.

2.9.2 Phytotoxicity of RDX

Range-finding tests were performed in which each of the three plant species was exposed to nominal RDX concentrations in the five soils to identify the range of concentrations to use in the limit tests. Nominal RDX concentrations used in the range-finding tests were 0 (no amendment), 5000, and 10,000 mg kg^{-1}. Planting procedures and cultural practices for the range-finding tests were the same as those described in Section 2.9, "Phytotoxicity Assessment".

Limit tests were performed on the basis of the range-finding test results. Treatment levels for the limit tests were 0 (no amendment), 0 (acetone carrier), and 10,000 mg kg^{-1} RDX. Eight replicates of each plant species were tested for each treatment level in each of the five soils.

Definitive tests were performed with each of the three plant species using treatment levels that were selected on the basis of the range-finding and limit test results. Nominal treatment concentrations were 0 (no amendment), 0 (acetone carrier), 100, 300, 600, 1200, 2500, and 5000 mg kg^{-1} RDX in the TSL, SSL, and KCL soils. Each of the three plant species was separately exposed to nominal RDX concentrations of 0 (no amendment), 0 (acetone carrier), and 300, 600, 1200, 2500, and 5000 mg kg^{-1} in the RCL and WCL soils.

2.9.3 Positive Control

Positive-control treatments were included in each definitive toxicity test with either TNT or RDX for each soil and each species (alfalfa, J. millet, and perennial ryegrass). The reference toxicant used for the positive control in definitive toxicity tests in these studies was boric acid (H_3BO_3). Preliminary tests were performed with alfalfa and perennial ryegrass in TSL and KCL soils amended with boric acid to determine the concentration that produced a 50% reduction in the selected measurement endpoint (EC_{50} value); in these tests, the measurement endpoint was SDM. The nominal concentrations of boric acid used in these preliminary studies were 0 (no amendment), 10, 20, 40, 80, 160, 320, and 640 mg kg^{-1}; each concentration was replicated three times. Nonlinear regression analysis (described in Section 2.10, "Data Analysis") was used to determine the respective EC_{50} values. The EC_{50} values determined in these preliminary tests were applied as the appropriate positive-control treatment levels for the definitive tests. The EC_{50} value that was determined for alfalfa in TSL soil (99 mg kg^{-1}) was used as the positive-control treatment level in the definitive tests with alfalfa in TSL and SSL soils. The EC_{50} value that was determined for perennial ryegrass in TSL soil (77 mg kg^{-1}) was used as the positive-control treatment level in the definitive tests with perennial ryegrass and J. millet in TSL and SSL soils, respectively. The EC_{50} value that was determined for alfalfa in KCL soil (264 mg kg^{-1}) was used as the positive-control treatment level in the definitive tests with alfalfa in KCL, RCL, and WCL soils. The EC_{50} value that was determined for perennial ryegrass in KCL soil (212 mg kg^{-1}) was used as the positive-control treatment level in the definitive tests with perennial ryegrass and J. millet in KCL, RCL, and WCL soils.

2.10 Data Analysis

Seedling emergence values and the respective SFM and SDM data were analyzed independently for each species using nonlinear or linear regression models as described in Stephenson et al. (2000) and Kuperman et al. (2003). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to determine whether to weight the data and to help select the type of regression model to be used for each data set. The models selected had the best fit of the data points to curves generated by the respective models, the smallest variances , and the residuals with the best appearance (i.e., most random scattering). The models selected for data comparison in these studies were:

Logistic (Gompertz) model: $$Y = a \times e^{\{[\log(1-p)] \times [C/\mathrm{ECp}]b\}} \quad (1)$$

Exponential model: $$Y = a \times e^{(\{[\log(1-p)]/\mathrm{ECp}\} \times C) + b} \quad (2)$$

Hormetic model: $$Y = a \times [1 + (h \times C)]/\{1 + [(p + (h \times C))/(1-p)] \times [C/\mathrm{ECp}]^b\} \quad (3)$$

Linear model: $$Y = [(-a \times p)/\mathrm{ECp}] \times C + a \quad (4)$$

where

- Y is the measurement endpoint;
- a is the y-intercept (e.g., control response);
- e is the base of the natural logarithm;
- p is the percent inhibition/100 (e.g., 0.5 for EC_{50});
- C is the test concentration;
- ECp is the estimate of effective concentration for a specified percent effect;
- b is the scale parameter; and
- h is the hormetic effect parameter.

Data that exhibited hormesis, a concentration-response phenomenon characterized by low-dose stimulation and high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The ECp parameters used in this study included the TNT and RDX concentrations that produced 20% (EC_{20}) and 50% (EC_{50}) reductions in the measurement endpoints compared with carrier controls. The EC_{20} parameter based on a growth endpoint is the preferred parameter for deriving plant Eco-SSL benchmarks (U.S. EPA, 2005). The EC_{50} values (more commonly used in the past) were included to enable comparisons of the results produced in these studies with those reported by other researchers.

Analysis of variance (ANOVA) was used to determine the no-observed-effect (NOEC) and lowest-observed-effect (LOEC) concentration values. Mean separations were determined using Fisher's least-significant difference (FLSD) pairwise-comparison tests. A significance level of $p \leq 0.05$ was used to determine NOEC and LOEC values. Pearson's correlation analysis was used to estimate the contributions of OM, clay content, and pH to the relative toxicities of TNT or RDX to the three plant species in the five soils.

All statistical analyses were performed on untransformed toxicity data and analytically determined EM concentrations using SYSTAT 11.0 (Systat Software; Chicago, IL).

3. RESULTS

3.1 Measurement of pH in Soils Amended with TNT or RDX

Results of pH analyses are presented in Table 2. The pH values for soils amended with TNT did not vary greatly from the control soils. Slightly greater variation in pH occurred for soils amended with RDX; however, the standard error (SE) across treatments was only 0.06 pH units.

Table 2. Mean pH Values at the Start of Definitive Plant Testing with TNT or RDX W-A in Five Natural Soils

Nominal Concentration (mg kg^{-1})	Mean pH (n = 3)									
	TSL Soil		SSL Soil		KCL Soil		RCL Soil		WCL Soil	
	TNT	RDX	TNT	RDX	TNT	RDX	TNT	RDX	TNT	RDX
0	4.85	4.79	5.20	5.23	6.30	5.44	7.56	7.63	6.08	6.54
1	4.79	—	5.23	—	6.31	—	7.59	—	—	—
10	4.81	—	5.21	—	6.33	—	7.61	—	6.11	—
20	4.82	—	5.28	—	6.38	—	7.62	—	6.09	—
40	4.78	—	5.22	—	6.45	—	7.64	—	6.06	—
80	4.71	—	5.20	—	6.50	—	7.65	—	6.09	—
100	—	4.90	—	5.27	—	5.46	—	ND	—	ND
160	4.69	—	5.21	—	6.56	—	7.65	—	6.03	—
300	—	4.91	—	5.39	—	5.33	—	7.63	—	6.66
320	4.71	—	5.20	—	6.55		7.67	—	6.07	—
600	—	4.96	—	4.97	—	5.39	—	7.69	—	6.52
640	—	—	—	—	—	—	—	—	5.98	—
1200	—	4.90	—	4.96	—	5.38	—	7.65	—	6.62
2500	—	4.87	—	5.16	—	5.65	—	7.70	—	6.66
5000	—	4.90	—	5.12	—	5.53	—	7.73	—	6.67
	—	—	—	—	—	—	—	—	—	—
Mean	4.77	4.89	5.22	5.16	6.42	5.45	7.62	7.67	6.06	6.61
SE	0.02	0.02	0.01	0.06	0.04	0.04	0.01	0.02	0.02	0.03

—, Treatment level not used.
ND, not determined.

3.2 Analytical Determination of TNT in Soil

3.2.1 TNT in TSL Soil

Mean values of ACN-extractable TNT W-A for 70 days in TSL soil, expressed as percentages of amendments, ranged from below the detection limit (BDL) of 0.5 mg kg^{-1} at nominal 1 mg kg^{-1} to 80% at nominal 320 mg kg^{-1} (Table 3). Mean values of ATCLP-extractable TNT W-A in TSL soil ranged from BDL to 80% of ACN-extractable concentrations (Table 3).

Table 3. Analytically Determined Concentrations of TNT W-A for 70 Days in TSL Soil Used in Definitive Toxicity Tests with Plants

Nominal TNT Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1	BDL	BDL	BDL	BDL	BDL	BDL
10	1	0.01	10	BDL	BDL	BDL
20	3	0.1	14	1	0.1	33
40	15	0.2	37	8	0.1	53
80	45	0.3	56	31	0.4	69
160	116	1.0	72	94	0.3	81
320	254	2.0	80	204	2.2	80

Notes:
1. Analytically determined concentrations (nominal and average values; $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
2. Detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil.

3.2.2 TNT in SSL Soil

Mean values of ACN-extractable TNT W-A for 57 days in SSL soil, expressed as percentages of amendments, ranged from BDL of 0.5 mg kg^{-1} at nominal 1 mg kg^{-1} to 93% at nominal 320 mg kg^{-1} (Table 4). Mean values of ATCLP-extractable TNT W-A in SSL soil ranged from BDL to 99% of ACN-extractable concentrations (Table 4).

Table 4. Analytically Determined Concentrations of TNT W-A for 57 Days in SSL Soil Used in Definitive Toxicity Tests with Plants

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1	BDL	BDL	BDL	BDL	BDL	BDL
10	4	0.2	40	2	0.05	50
20	7	0.1	35	4	0.05	57
40	24	0.4	60	17	0.1	71
80	64	0.8	80	55	0.8	86
160	137	1.3	86	122	1.0	89
320	298	13.8	93	295	7.2	99

Notes:
1. Analytically determined concentrations (nominal and average values; $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
2. Detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil.

3.2.3 TNT in KCL Soil

Mean values of ACN-extractable TNT W-A for 59 days in KCL soil, expressed as percentages of amendments, ranged from BDL of 0.5 mg kg^{-1} at nominal 1 mg kg^{-1} to 35% at nominal 320 mg kg^{-1} (Table 5). Mean values of ATCLP-extractable TNT W-A in KCL soil ranged from BDL to 68% of ACN-extractable values (Table 5).

Table 5. Analytically Determined Concentrations of TNT W-A for 59 Days in KCL Soil Used in Definitive Toxicity Tests with Plants

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1	BDL	BDL	BDL	BDL	BDL	BDL
10	1	0.02	10	0.3	0.03	30
20	4	0.3	20	1	0.03	25
40	11	0.07	28	4	0.1	36
80	25	0.2	31	13	0.2	52
160	42	0.7	26	27	0.1	64
320	112	6.6	35	76	1.3	68

Notes:
1. Analytically determined concentrations (nominal and average values; $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
2. Detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil.

3.2.4 TNT in RCL Soil

Mean values of ACN-extractable TNT W-A for 56 days in RCL soil, expressed as percentages of amendments, ranged from BDL of 0.5 mg kg^{-1} at nominal 1–20 mg kg^{-1} to 38% at nominal 320 mg kg^{-1} (Table 6). Mean values of ATCLP-extractable TNT W-A in RCL soil ranged from BDL to 60% of ACN-extractable values (Table 6).

Table 6. Analytically Determined Concentrations of TNT W-A for 56 Days in RCL Soil Used in Definitive Toxicity Tests with Plants

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
1	BDL	BDL	BDL	BDL	BDL	BDL
10	BDL	BDL	BDL	BDL	BDL	BDL
20	BDL	BDL	BDL	BDL	BDL	BDL
40	0.6	0.09	1.5	BDL	BDL	BDL
80	3	0.09	4	0.95	0.04	32
160	39	0.3	24	19	0.3	49
320	121	2.0	38	73	0.4	60

Notes:
1. Analytically determined concentrations (nominal and average values; $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
2. Detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil.

3.2.5 TNT in WCL Soil

Mean values of ACN-extractable TNT W-A for 55 days in WCL soil, expressed as percentages of amendments, ranged from BDL of 0.5 mg kg^{-1} at nominal 0 mg kg^{-1} to 80% at nominal 640 mg kg^{-1} (Table 7). Mean values of ATCLP-extractable TNT W-A in WCL soil ranged from BDL to 49% of ACN-extractable values (Table 7).

Table 7. Analytically Determined Concentrations of TNT W-A for 55 Days in WCL Soil Used in Definitive Toxicity Tests with Plants

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	BDL	BDL	BDL	BDL
10	1.5	0.15	15	BDL	BDL	BDL
20	4.5	0.6	23	BDL	BDL	BDL
40	9	2.1	23	BDL	BDL	BDL
80	26	1.9	33	1	0.1	4
160	78	4.7	49	9	0.3	12
320	194	13.5	61	53	4.8	27
640	514	6.6	80	251	13.9	49

Notes:
1. Analytically determined concentrations (nominal and average values; $n = 3$) included ACN-extractable (U.S. EPA Method 8330A) and water-extractable (ATCLP; Haley et al., 1993) concentration values.
2. Detection limits were 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil.

3.3 Phytotoxicity of TNT in Five Natural Soils

3.3.1 Range-Finding Plant Toxicity Tests with TNT

Nominal TNT concentrations used in the range-finding tests were 0 (no amendment), 10, 100, 200, 400, and 800 mg kg^{-1}. For most soils treated with TNT concentrations ≥200 mg kg^{-1}, seedling emergence values for most species were significantly ($p < 0.05$) reduced as compared with the carrier controls. The exceptions were J. millet and perennial ryegrass (91%) in WCL soil, perennial ryegrass in TSL soil (97%), and J. millet in RCL soil, which had no significant reduction ($p > 0.05$) in seedling emergence values in soils containing nominal TNT concentrations up to and including 800 mg kg^{-1}. SDM, in relation to nominal TNT concentrations in soil, differed among species and soil type. The LOEC ($p < 0.05$) values for alfalfa SDM in soil amended with TNT were 100 mg kg^{-1} in TSL; 400 mg kg^{-1} in SSL; and 100 mg kg^{-1}in RCL, KCL, and WCL soils. For J. millet, the LOEC values were 200 mg kg^{-1} in TSL, 100 mg kg^{-1} in SSL, 200 mg kg^{-1} in KCL and RCL, and 10 mg kg^{-1} in WCL. For perennial ryegrass, the LOEC values were 100 mg kg^{-1} in TSL, 200 mg kg^{-1} in SSL, 100 mg kg^{-1} in KCL and RCL, and 400 mg kg^{-1} in WCL (Figure 3). Seedling emergence values and SDM data were used to select nominal TNT concentrations for the definitive toxicity tests. Selected nominal and resulting ACN-extractable concentrations of TNT used in the definitive toxicity tests are shown in Section 3.2, "Analytical Determination of TNT in Soil", for all treatment levels in each of the five natural soils.

3.3.2 Definitive Phytotoxicity Tests with TNT

Independent definitive studies were conducted using methods adapted from the standardized protocols, *Standard Guide for Conducting Terrestrial Plant Toxicity Tests* (ASTM, 2002) and *Ecological Effects Test Guidelines: Early Seedling Growth Toxicity Test* (U.S. EPA, 1996), to assess the effects of TNT on the alfalfa, perennial ryegrass, and J. millet test species in TSL, SSL, KCL, RCL, and WCL soils. Seedling emergence, SFM, and SDM values were

assessed with respect to EM concentrations in soil, which were selected on the basis of the results from the range-finding studies. Ecotoxicological benchmark values were established utilizing plant responses for toxicological endpoints to TNT concentrations in soil that were analytically determined using EPA Method 83330A (U.S. EPA, 2007). Test results complied with the validity criteria for the test: seedling emergence values in the control groups were at least 75% of the total number of seeds planted, and mean survival rates for control seedlings were at least 75% at the end of the test. Data from these tests fit linear, logistic (Gompertz), exponential, or hormetic models (Table 8) (Stephenson et al., 2000).

In all of the soils tested, TNT was relatively toxic to plants (Table 8). On the basis of the EC_{20} and EC_{50} values and the corresponding 95% confidence intervals (CIs), plant growth endpoints (SFM and SDM) were more-sensitive indicators of TNT toxicity than were seedling emergence values (Table 8). The EC_{20} values for plant growth, calculated using ACN-extractable concentrations of TNT in soil, ranged from 5 mg kg^{-1} (for SFM of J. millet in SSL soil and SDM of perennial ryegrass in TSL soil) to 137 mg kg^{-1} (for SFM of perennial ryegrass in WCL soil). Seedling emergence EC_{20} values for TNT ranged from 40 mg kg^{-1} (for alfalfa in KCL soil) to 339 mg kg^{-1} (for perennial ryegrass in WCL soil). Seedling emergence for J. millet was not affected by TNT in KCL soil, up to and including TNT concentrations of 112 mg kg^{-1}. On the basis of the 95% CIs for the respective EC_{20} and EC_{50} SDM values, the resulting toxicity of TNT W-A in soil to all three plant species was not significantly different among the TSL, SSL, KCL, and RCL soils. However, toxicity to all tested species was significantly less when plants were grown in WCL soil, which has greater amounts of OM and clay than the other soils tested.

The concentration-response relationships for TNT in soil and the production of SDM by alfalfa, J. millet, and perennial ryegrass in the five soils tested are shown in Figures 1, 2, and 3. The SDM variable was chosen to show the differences among species and soils because the majority of plant dry matter consists of assimilates (i.e., carbohydrates, proteins, and lipids) that are synthesized during photosynthesis (Natr and Lawlor, 2005). Data were fit to logistic Gompertz, exponential, logistic hormetic, or linear models on the basis of the best-fit criteria described in Section 2.10, “Data Analysis”.

Table 8. Summary of Toxicological Benchmark Concentrations for TNT Independently W-A in TSL, SSL, KCL, RCL, and WCL Soils, Determined for Alfalfa, J. Millet, and Perennial Ryegrass

Soil Type	Seedling Emergence			SFM			SDM		
	Regression Model	EC_{20} (mg kg^{-1})	EC_{50} (mg kg^{-1})	Regression Model	EC_{20} (mg kg^{-1})	EC_{50} (mg kg^{-1})	Regression Model	EC_{20} (mg kg^{-1})	EC_{50} (mg kg^{-1})
Alfalfa									
TSL	Linear	54 (44–64)	135 (109–161)	Hormetic	12 (1–22)	36 (10–62)	Hormetic	18 (3–33)	42 (3–81)
SSL	Gompertz	68 (40–96)	84 (77–91)	Exponential	7 (4–11)	22 (12–33)	Exponential	10 (4–16)	31 (13–49)
KCL	Linear	40 (28–52	99 (70–129)	Hormetic	9 (4–13)	20 (11–29)	Hormetic	13 (8–17)	26 (17–34
RCL	Linear	43 (26–61)	108 (64–152)	Hormetic	21 (8–33)	33 (20–46)	Hormetic	8 (3–13)	22 (1–43)
WCL	Linear	254 (143–365)	635 (358–911)	Hormetic	114 (80–149)	200 (129–270)	Hormetic	113 (79–148)	206 (135–278)
J. Millet									
TSL	Linear	65 (55–76)	163 (137–190)	Hormetic	21 (7–34)	40 (14–65)	Gompertz	28 (6–50)	56 (32–79)
SSL	Gompertz	67 (35–99)	169 (131–206)	Hormetic	5 (4–6)	8 (6–9)	Hormetic	6 (5–7)	10 (8–12)
KCL	NS	NS	NS	Hormetic	12 (9–14)	16 (13–20)	Hormetic	12 (10–14)	20 (18–23)
RCL	Linear	68 (52–85)	171 (129–212)	Hormetic	15 (0–32)	26 (17–36)	Hormetic	11 (0–23)	22 (14–31)
WCL	Linear	266 (190–343)	666 (475–858)	Hormetic	105 (89–122)	157 (130–183)	Hormetic	99 (86–111)	147 (128–167)
Perennial Ryegrass									
TSL	Gompertz	58 (41–76)	92 (80–105)	Exponential	8 (4–12)	24 (12–37)	Gompertz	5 (0–10)	23 (11–36)
SSL	Gompertz	27 (16–38)	56 (45–67)	Hormetic	7 (5–8)	11 (9–13)	Hormetic	7 (5–8)	11 (8–14)
KCL	Linear	44 (37–51)	110 (93–129)	Hormetic	10 (9–12)	16 (14–17)	Hormetic	12 (8–14)	20 (17–22)
RCL	Linear	40 (35–45)	100 (88–113)	Hormetic	7 (2–12)	11 (9–13)	Exponential	9 (5–13)	28 (17–40)
WCL	Gompertz	339 (238–440)	480 (443–518)	Gompertz	137 (64–212)	184 (161–206)	Hormetic	127 (90–164)	185 (144–225)

Note: 95% CIs are shown in parentheses.
NS, not significant at $p > 0.05$.

Alfalfa

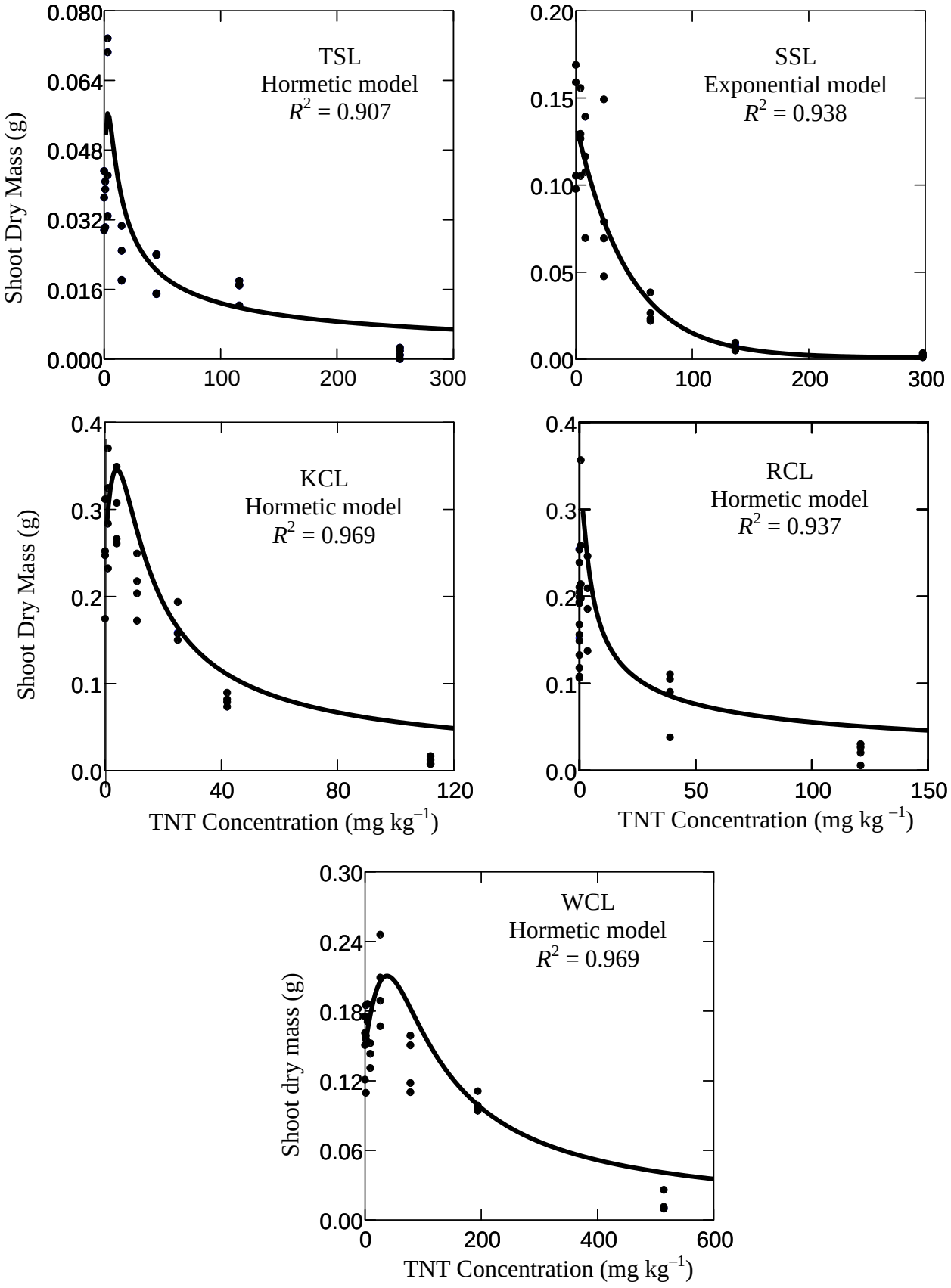

Figure 1. Exposure-response curves showing the relationship between ACN-extractable concentrations of TNT W-A in five natural soils and SDM of alfalfa. R^2, regression sum of squares divided by total sum of squares.

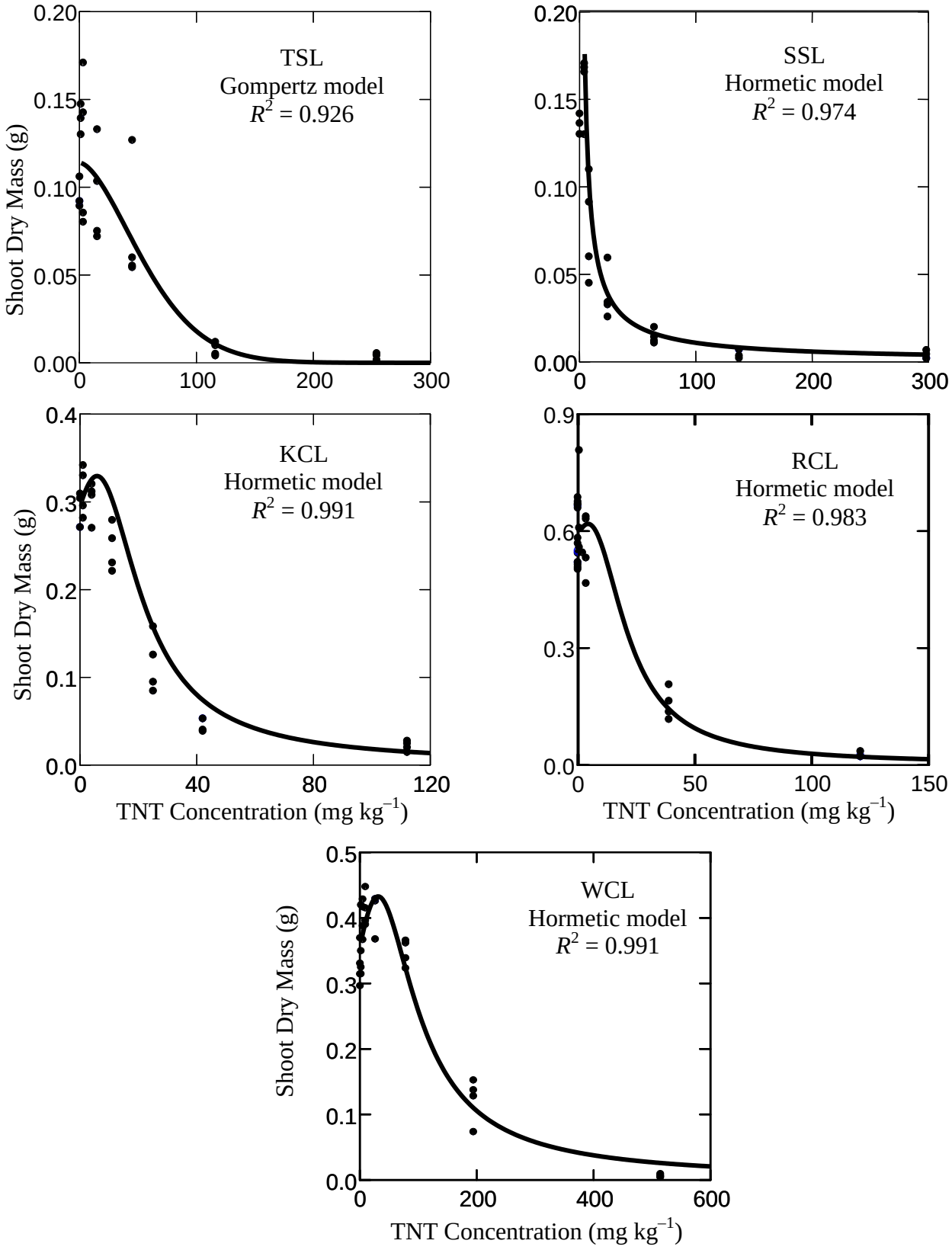

Figure 2. Exposure-response curves showing the relationship between ACN-extractable concentrations of TNT W-A in five natural soils and SDM of J. millet.

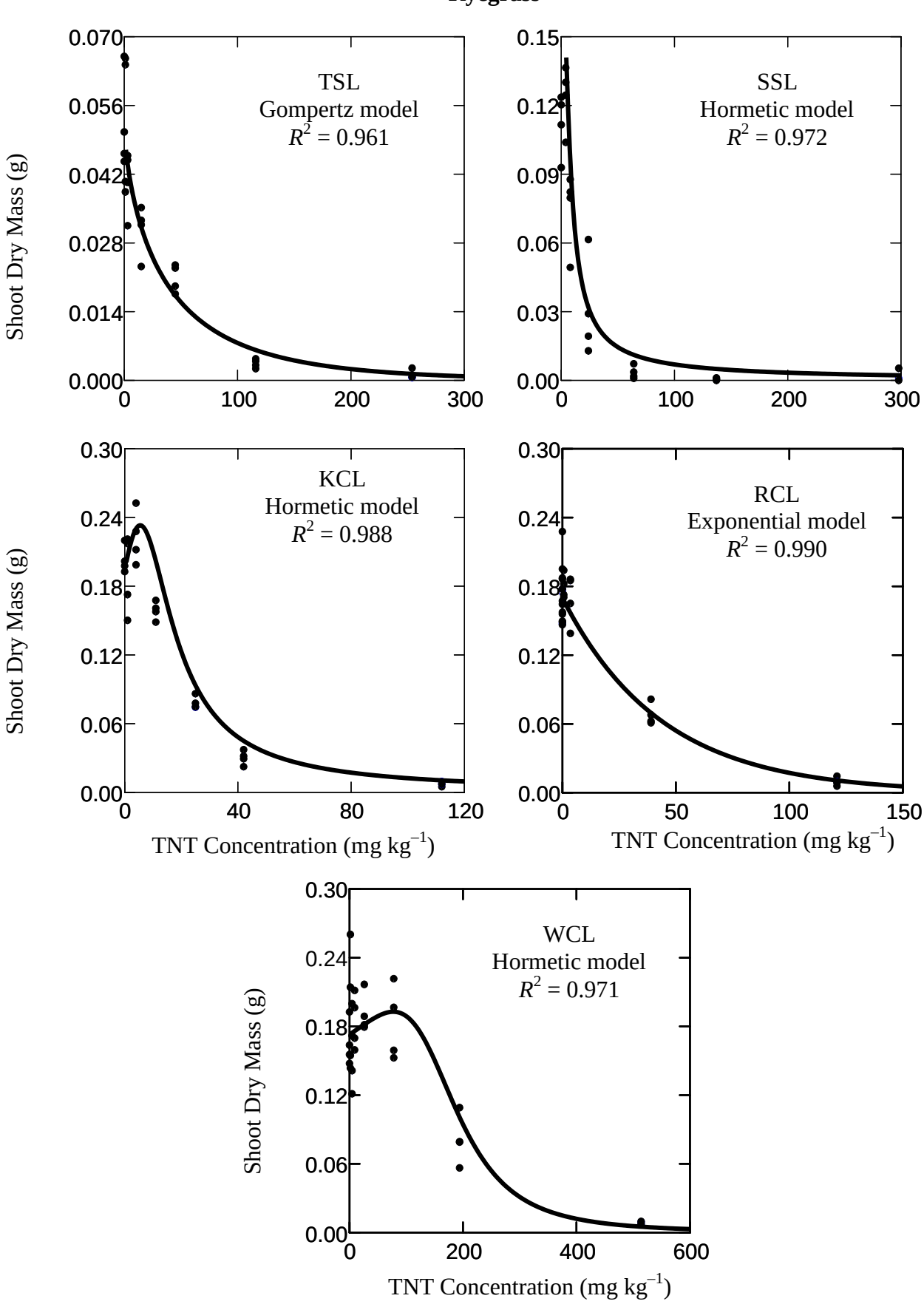

Figure 3. Exposure-response curves showing the relationship between ACN-extractable concentrations of TNT W-A in five natural soils and SDM of perennial ryegrass.

3.3.3 Effects of Selected Soil Properties on Phytotoxicity of TNT

Pearson's correlation analysis was used to determine the relationship between selected soil properties and EC_{50} values for the SDM response to TNT W-A in the five soils. None of the soil properties were significantly ($p \leq 0.05$) correlated with EC_{50} values for SDM for any of the three species (Table 9), although the Pearson's correlation coefficient (*r*-value) for OM was relatively high for the three species when compared with corresponding *r*-values for clay and pH.

Table 9. Pearson's Correlation Coefficients and Probability Values for TNT Toxicity Endpoints (EC_{50} Levels) for SDM of Alfalfa, J. Millet, or Perennial Ryegrass and Selected Soil Properties

Soil Property	Alfalfa SDM (EC_{50})		J. Millet SDM (EC_{50})		Perennial Ryegrass SDM (EC_{50})	
	r	*p*	*r*	*p*	*r*	*p*
OM (%)	0.821	0.089	0.770	0.128	0.875	0.052
Clay (%)	0.318	0.602	0.230	0.710	0.430	0.470
pH	–0.060	0.924	–0.154	0.804	0.037	0.953

3.4 Analytical Determination of RDX in Soil

RDX concentrations in soil remained relatively close to nominal levels, with little apparent transformation or fixation in each soil after 3 months of weathering-and-aging in the limit and definitive tests (Tables 10 and 11, respectively). Mean values for ACN-extractable RDX W-A for 3 months in soils for the limit tests, expressed as percentages of nominal concentrations, ranged from 83% in TSL soil with alfalfa or J. millet to 116% in TSL soil with perennial ryegrass (Table 10). Mean values of ACN-extractable RDX W-A for 3 months in soils used in the definitive tests, expressed as percentages of nominal concentrations, ranged from 91% at nominal 300 mg kg^{-1} in RCL and 600 mg kg^{-1} in WCL to 114% at nominal 5000 mg kg^{-1} in TSL (Table 11).

Table 10. Seedling Emergence, SFM, and SDM of Alfalfa, J. Millet, and Perennial Ryegrass Grown in Five Natural Soils Amended with RDX W-A for 3 Months, Determined in Limit Tests

RDX Concentration (mg kg^{-1})		Species	Seedling Count		SFM		SDM	
Nominal	Measured		No.	SE	g	SE	g	SE
SSL Soil								
0	BDL	Alfalfa	15.6 (78%)	2.1	0.72	0.111	0.20	0.026
10,000	9,929	Alfalfa	15.6 (78%)	1.5	0.65	0.025	0.15	0.006
0	BDL	J. millet	18.0 (90%)	0.3	2.79	0.063	0.46	0.013
10,000	9,929	J. millet	18.5 (93%)	0.3	**2.11**[a]	0.074	**0.36**[a]	0.014
0	BDL	Ryegrass	18.6 (93%)	0.5	1.73	0.065	0.32	0.016
10,000	9,929	Ryegrass	18.9 (95%)	0.4	**1.16**[a]	0.048	**0.23**[a]	0.007
TSL Soil								
0	BDL	Alfalfa	14.4 (72%)	1.1	0.14	0.015	0.06	0.005
10,000	8,320	Alfalfa	14.5 (73%)	1.1	0.15	0.018	0.06	0.004
0	BDL	J. millet	17.6 (88%)	0.5	0.35	0.038	0.07	0.006
10,000	8,320	J. millet	16.3 (82%)	0.6	**0.15**[a]	0.014	**0.03**[a]	0.004
0	BDL	Ryegrass	18.6 (93%)	0.5	0.37	0.028	0.07	0.005
10,000	11,641	Ryegrass	18.9 (95%)	0.4	**0.12**[a]	0.007	**0.04**[a]	0.003
KCL Soil								
0	BDL	Alfalfa	15.0 (75%)	1.9	0.77	0.051	0.17	0.008
10,000	9353	Alfalfa	13.6 (68%)	2.2	0.48	0.074	0.10	0.010
0	BDL	J. millet	17.6 (88%)	1.0	1.44	0.080	0.27	0.016
10,000	9353	J. millet	17.5 (88%)	0.6	1.26	0.111	0.26	0.020
0	BDL	Ryegrass	18.4 (92%)	0.3	1.10	0.031	0.20	0.004
10,000	11,287	Ryegrass	17.9 (90%)	0.8	**0.55**[a]	0.055	**0.11**[a]	0.012
RCL Soil								
0	BDL	Alfalfa	14.1 (71%)	1.4	1.98	0.116	0.37	0.024
10,000	10,046	Alfalfa	12.8 (64%)	0.7	**1.43**[a]	0.119	**0.26**[a]	0.017
0	BDL	J. millet	17.1 (86%)	0.6	4.97	0.195	0.75	0.047
10,000	10,046	J. millet	17.6 (88%)	0.6	**4.05**[a]	0.186	**0.60**[a]	0.033
0	BDL	Ryegrass	17.5 (88%)	0.6	1.90	0.061	0.31	0.011
10,000	10,046	Ryegrass	19.0 (95%)	0.4	**1.19**[a]	0.052	**0.22**[a]	0.010
WCL Soil								
0	BDL	Alfalfa	17.5 (88%)	0.7	1.40	0.074	0.29	0.015
10,000	8,811	Alfalfa	15.1 (76%)	0.8	**0.78**[a]	0.041	**0.15**[a]	0.007
0	BDL	J. millet	17.6 (88%)	0.4	3.02	0.125	0.49	0.028
10,000	8,811	J. millet	18.0 (90%)	0.4	**2.58**[a]	0.107	**0.41**[a]	0.024
0	BDL	Ryegrass	18.8 (94%)	0.4	1.65	0.045	0.28	0.007
10,000	8,811	Ryegrass	18.1 (91%)	0.4	**0.93**[a]	0.061	**0.16**[a]	0.011

Note: Values are averages (n = 8).
[a]Significant decrease ($p < 0.05$) compared with control, shown in bold print.
BDL, below detection limit of 0.05 mg kg^{-1}.

Table 11. Nominal and Average (n = 3) Analytically Determined Concentrations of RDX W-A in Five Soils Used in the Definitive Toxicity Tests with Plants

Soil Type	Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/Nominal (%)
TSL	0	BDL	BDL	BDL
	100	100	2.1	100
	300	311	4.4	104
	600	608	5.1	102
	1200	1183	2.7	99
	2500	2655	31.4	106
	5000	5687	82.0	114
SSL	0	BDL	BDL	BDL
	100	94	3.7	94
	300	321	12.4	107
	600	609	15.7	102
	1200	1149	9.9	96
	2500	2511	25.5	100
	5000	5211	31.4	104
KCL	0	BDL	BDL	BDL
	100	99	2.9	99
	300	287	8.0	96
	600	584	12.6	97
	1200	1135	33.5	95
	2500	2442	28.5	98
	5000	5031	37.9	101
RCL	0	BDL	BDL	BDL
	300	273	6.7	91
	600	596	20.2	99
	1200	1198	9.0	100
	2500	2460	48.8	98
	5000	5157	97.2	103
WCL	0	BDL	BDL	BDL
	300	296	10.7	99
	600	548	28.7	91
	1200	1182	14.7	99
	2500	2487	65.2	99
	5000	5191	566.1	104

BDL, below detection limit of 0.5 mg kg^{-1}.

3.5 Phytotoxicity of RDX in Five Natural Soils

3.5.1 Phytotoxicity of RDX: Range-Finding Tests

Seedling emergence values for all plant species were not significantly reduced ($p > 0.01$) after exposure to nominal FA RDX concentrations of 5,000 and 10,000 mg kg^{-1} compared with unamended controls in any of the five soils in this study. Mean SDM values for the test plants exposed to RDX in the five soils are shown in Figure 4. Mean SDM values for perennial ryegrass were significantly ($p < 0.05$) decreased compared with control soil values at nominal RDX concentrations of 5,000 and 10,000 mg kg^{-1} in TSL soil, and 10,000 mg kg^{-1} in

KCL soil (Figure 4). Significant ($p < 0.05$) growth inhibition (on the basis of SDM values) by exposure to RDX was also determined at 10,000 mg kg^{-1} for J. millet in WCL soil and alfalfa in RCL soil (Figure 4).

3.5.2 Phytotoxicity of RDX: Limit Tests

On the basis of the range-finding test results, individual limit tests were performed for each species in each soil type. Results of the limit tests are provided in Table 10. Mean SFM and SDM values were significantly reduced ($p < 0.05$) for alfalfa in RCL and WCL soils; for J. millet in TSL, SSL, RCL, and WCL soils; and for perennial ryegrass in all five soils. Seedling emergence values were not significantly reduced compared with controls ($p > 0.05$) for any of the plant species tested in the five soils (Table 10).

3.5.3 Phytotoxicity of RDX: Definitive Tests

Definitive plant toxicity tests were designed as described in Section 2.9.2, "Phytotoxicity of RDX". Results of statistical analyses by ANOVA for NOEC and LOEC values are shown in Table 12. Seedling emergence values for alfalfa, J. millet, and perennial ryegrass were not negatively affected by soil RDX concentrations in any of the soils tested. The effects of RDX on SFM and SDM were variable with respect to plant species and soil. The SFM and SDM values for alfalfa were not significantly ($p > 0.05$) different between the control treatments and any positive RDX treatments in all soils tested in the definitive plant toxicity tests (Table 12). On the basis of NOEC and LOEC values for SFM and SDM, soil RDX concentrations were phytotoxic to J. millet in TSL, SSL, RCL, and WCL soils. The respective NOEC and LOEC values for RDX in soil, based on the SFM of J. millet, were 311 and 608 mg kg^{-1} in TSL, <94 and 94 mg kg^{-1} in SSL, <273 and 273 mg kg^{-1} in RCL, and <296 and 296 mg kg^{-1} in WCL (Table 12). The respective NOEC and LOEC values for RDX in soil, based on the SDM for J. millet, were 100 and 311 mg kg^{-1} in TSL, <94 and 94 mg kg^{-1} in SSL, <273 and 273 mg kg^{-1} in RCL, and <296 and 296 mg kg^{-1} in WCL (Table 12). On the basis of NOEC and LOEC values for SFM and SDM, the RDX concentrations in KCL soil were not phytotoxic to J. millet (Table 12). Perennial ryegrass was the most sensitive species to RDX in this study. The respective NOEC and LOEC values for RDX in soil, based on the SFM for perennial ryegrass, were 311 and 608 mg kg^{-1} in TSL, 94 and 321 mg kg^{-1} in SSL, <99 and 99 mg kg^{-1} in KCL, <273 and 273 mg kg^{-1} in RCL, and <296 and 296 mg kg^{-1} in WCL (Table 12). The respective NOEC and LOEC values for RDX in soil, based on the SDM values for perennial ryegrass, were 311 and 608 mg kg^{-1} in TSL, 94 and 321 mg kg^{-1} in SSL, <99 and 99 mg kg^{-1} in KCL, <273 and 273 mg kg^{-1} in RCL, and <296 and 296 mg kg^{-1} in WCL, respectively (Table 12).

The results of regression analyses of soil RDX concentrations with seedling emergence, SFM, and SDM values for alfalfa, J. millet, and perennial ryegrass are shown in Table 13. The exponential model had the best fit for all data in which a regression analysis could be determined. The effects of RDX on J. millet SFM were dependent on soil type. J. millet SFM data yielded the respective EC_{20} and EC_{50} values of 100 and 310 mg kg^{-1} in TSL, and 55 and 172 mg kg^{-1} in WCL (Table 13); regression models did not adequately characterize SFM data for J. millet in SSL, KCL, and RCL soils (Table 13). The SDM data for J. millet in TSL, SSL, RCL, and WCL soils yielded the respective EC_{20} and EC_{50} values of 73 and 226 mg kg^{-1}, 33 and 104 mg kg^{-1}, 19 and 59 mg kg^{-1}, and 48 and 149 mg kg^{-1} (Table 13). Regression models did not

adequately characterize SDM data for J. millet in KCL soil. Perennial ryegrass was the most sensitive species overall to soil RDX, with SFM data yielding the respective EC_{20} and EC_{50} values of 91 and 281 mg kg^{-1} in TSL, 51 and 158 mg kg^{-1} in SSL, 10 and 32 mg kg^{-1} in KCL, 18 and 56 mg kg^{-1} in RCL, and 25 and 79 mg kg^{-1} in WCL (Table 13). On the basis of the SDM data for perennial ryegrass, the respective EC_{20} and EC_{50} values were 104 and 323 mg kg^{-1} for TSL, 78 and 242 mg kg^{-1} for SSL, 7 and 20 mg kg^{-1} for KCL, 16 and 49 mg kg^{-1} for RCL, and 37 and 115 mg kg^{-1} for WCL soils. Toxicity data for alfalfa in all soils did not fit into either nonlinear or linear exposure-response models. Seedling emergence values were not reduced by RDX for each of the three plant species in all three soils.

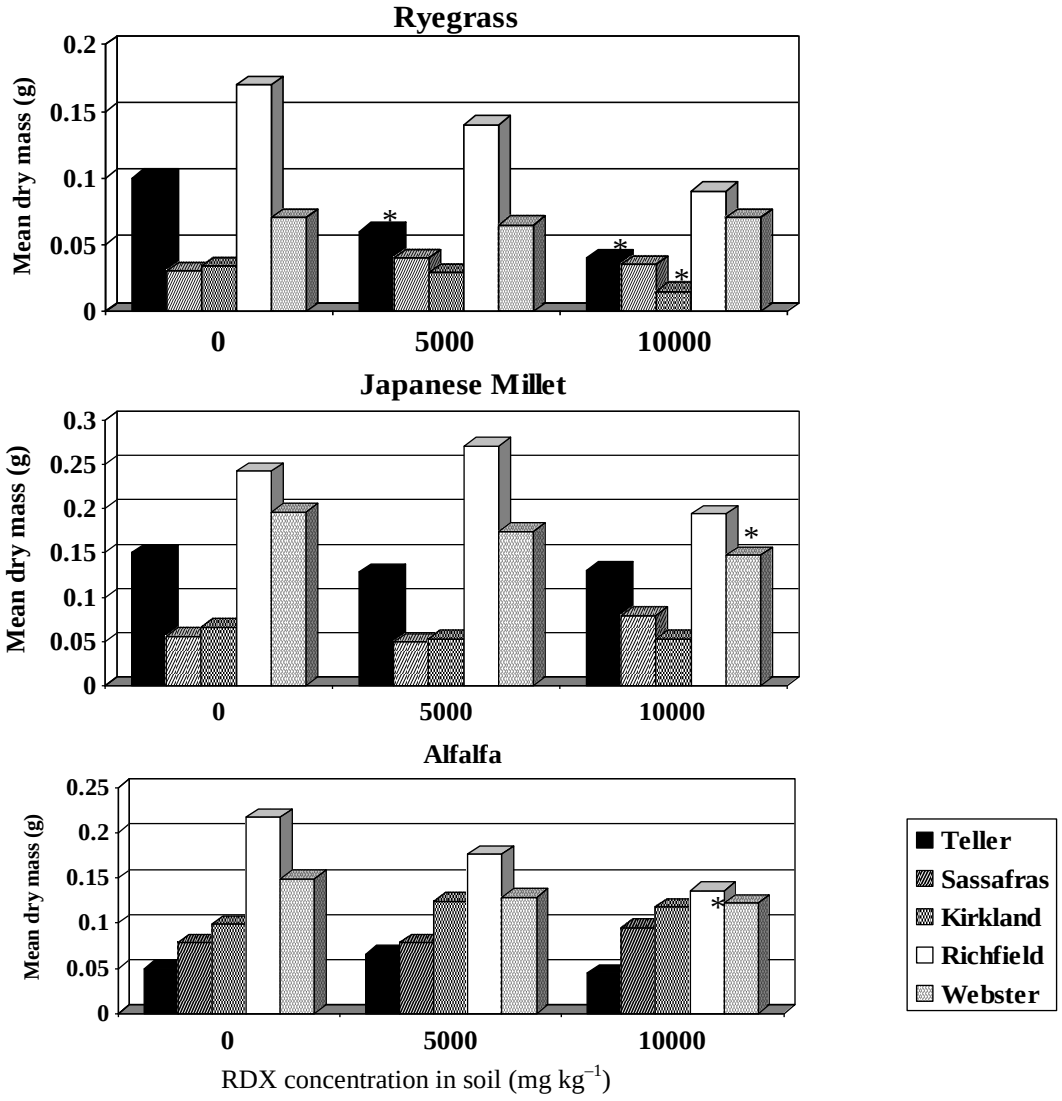

Figure 4. Mean SDM of perennial ryegrass, J. millet, and alfalfa exposed to RDX in range-finding toxicity tests (n = 2). *Significant decrease in SDM ($p < 0.05$) compared with control.

Table 12. Summary: NOEC and LOEC Values for Analytically Determined RDX W-A in TSL, SSL, RCL, KCL, and WCL Soils for Seedling Emergence, SFM, and SDM for Alfalfa, J. Millet, and Perennial Ryegrass in Definitive Toxicity Tests

Soil Type	Seedling Emergence				SFM				SDM			
	NOEC (mg kg^{-1})	*p*-Value	LOEC (mg kg^{-1})	*p*-Value	NOEC (mg kg^{-1})	*p*-Value	LOEC (mg kg^{-1})	*p*-Value	NOEC (mg kg^{-1})	*p*-Value	LOEC (mg kg^{-1})	*p*-Value
Alfalfa												
TSL	5687	>0.05	>5687	>0.05	5687	>0.05	>5687	>0.05	5687	>0.05	>5687	>0.05
SSL	5211	>0.05	>5211	>0.05	5211	>0.05	>5211	>0.05	5211	>0.05	>5211	>0.05
KCL	5031	>0.05	>5031	>0.05	5031	>0.05	>5031	>0.05	5031	>0.05	>5031	>0.05
RCL	5157	>0.05	>5157	>0.05	5157	>0.05	>5157	>0.05	5157	>0.05	>5157	>0.05
WCL	5191	>0.05	>5191	>0.05	5191	0.789	>5191	>0.05	5191	0.164	>5191	>0.05
J. Millet												
TSL	5687	>0.05	>5687	>0.05	311	0.109	608[a]	0.008	100	1.000	311[a]	0.002
SSL	5211	>0.05	>5211	>0.05	<94	1.000	94[a]	0.003	<94	1.000	94	<0.001
KCL	5031	>0.05	>5031	>0.05	5031	>0.05	>5031	>0.05	5031	>0.05	>5031	>0.05
RCL	5157	>0.05	>5157	>0.05	<273	1.000	273[a]	<0.001	<273	1.000	273[a]	<0.001
WCL	5191	>0.05	>5191	>0.05	<296	1.000	296[a]	<0.001	<296	1.000	296[a]	<0.001
Perennial Ryegrass												
TSL	5687	>0.05	>5687	>0.05	311	0.267	608[a]	0.017	311	0.501	608[a]	0.026
SSL	5211	>0.05	>5211	>0.05	94	0.086	321[a]	0.005	94	0.224	321[a]	0.007
KCL	5031	>0.05	>5031	>0.05	<99	1.000	99[a]	<0.001	<99	1.000	99[a]	<0.001
RCL	5157	>0.05	>5157	>0.05	<273	1.000	273[a]	<0.001	<273	1.000	273[a]	<0.001
WCL	5191	>0.05	>5191	>0.05	<296	1.000	296[a]	<0.001	<296	1.000	296[a]	<0.001

[a] Significant effect ($p < 0.05$) based on ANOVA and FLSD means-comparison analyses.

Table 13. Summary of Toxicological Benchmark Concentrations for RDX W-A in TSL, SSL, RCL, KCL, and WCL Soils for J. Millet and Perennial Ryegrass

Soil Type	SFM (mg kg^{-1})		SDM (mg kg^{-1})	
	EC_{20}	EC_{50}	EC_{20}	EC_{50}
J. Millet				
TSL	100 (2–197)	310 (7–613)	73 (30–115)	226 (94–358)
SSL	ND	ND	33 (10–57)	104 (30–177)
KCL	ND	ND	ND	ND
RCL	ND	ND	19 (0–62)	59 (0–193)
WCL	55 (14–96)	172 (44–300)	48 (22–74)	149 (68–231)
Perennial Ryegrass				
TSL	91 (0–204)	281 (0–633)	104 (0–237)	323 (0–737)
SSL	51 (4–98)	158 (11–304)	78 (0–157)	242 (0–486)
KCL	10 (5–16)	32 (15–50)	7 (0–17)	20 (0–53)
RCL	18 (0–65)	56 (0–201)	16 (0–78)	49 (0–242)
WCL	25 (1–50)	79 (2–157)	37 (10–64)	115 (32–197)

Note: 95% confidence intervals are shown in parentheses.
ND, not determined; data did not fit any of the regression models tested.

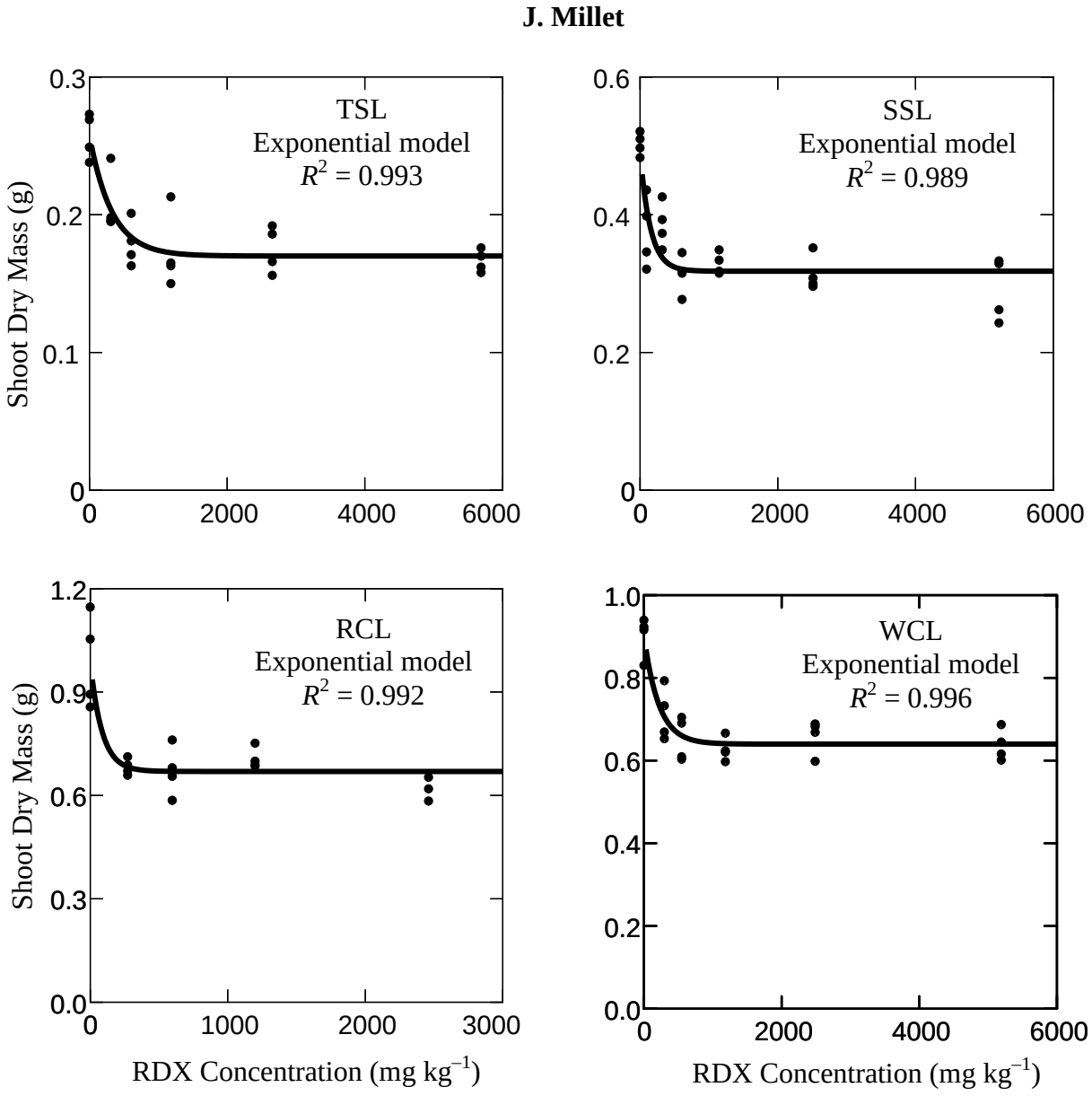

Figure 5. Exposure-response curves showing the relationship between ACN-extractable concentrations of RDX W-A in four natural soils and SDM of J. millet. RDX was not phytotoxic to J. millet in KCL soil; therefore, no regression models adequately fit these data.

Perennial Ryegrass

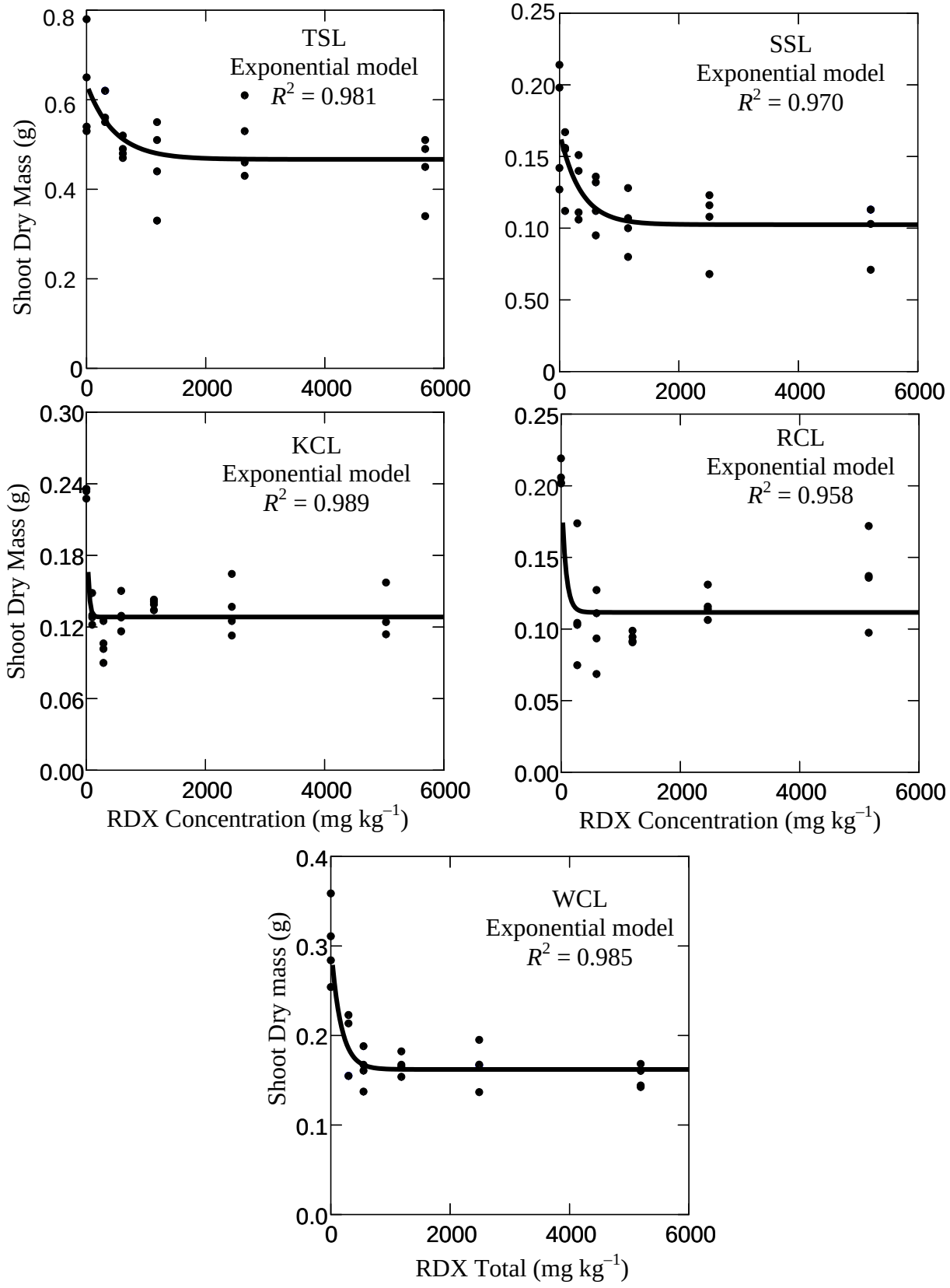

Figure 6. Exposure-response curves showing the relationship between ACN-extractable concentrations of RDX W-A in five natural soils and SDM of perennial ryegrass.

3.5.4 Effects of Soil Properties on Phytotoxicity of RDX

Results of Pearson's correlation analyses are shown in Table 14. A significant ($p \leq 0.05$) inverse correlation was identified between the RDX EC_{50} values for ryegrass SDM and both soil clay content ($r = –0.963$) and pH ($r = –0.915$). There was no significant correlation with OM. The RDX EC_{50} values for J. millet SDM were not significantly ($p \geq 0.05$) correlated with any of the three soil properties (Table 14). Alfalfa SDM was not reduced by RDX concentrations in soil in these studies; therefore, correlation analyses were not performed with this species.

Table 14. Pearson's Correlation Coefficients and Probability Values for RDX Toxicity Endpoints (EC_{50} Levels) for SDM of Perennial Ryegrass or J. Millet and Selected Soil Properties

Soil Property	Perennial Ryegrass SDM (EC_{50})		J. Millet SDM (EC_{50})	
	r	*p*	*r*	*p*
OM (%)	–0.582	0.303	–0.083	0.917
Clay (%)	–0.963[a]	0.008	–0.632	0.368
pH	–0.915[a]	0.030	–0.827	0.173

[a] Statistically significant ($p < 0.05$).

4. DISCUSSION

The goals of this research were to determine the ecotoxicological benchmarks for TNT and RDX for derivation of terrestrial, plant-based Eco-SSL values for use in ERAs of contaminated Superfund sites (U.S. EPA, 2005), and to investigate and characterize predominant soil physical and chemical parameters that may change the toxicological responses of the representative plant species tested to TNT or RDX. The majority of previous studies investigated hydroponic cultures or only one soil type and used freshly amended EMs rather than incorporating a weathering-and-aging procedure. In contrast, we conducted toxicity studies that produced phytotoxicity benchmarks for TNT and RDX that were W-A in natural soils from five soil series with differing physical and chemical characteristics. Application of the weathering-and-aging procedure to soils amended with a range of TNT or RDX concentrations allowed us to determine the net ecotoxicological effect of complex fate processes in soil that affect TNT and RDX bioavailability for selected terrestrial plant species. Testing under these conditions more closely resembled the environments encountered in the field. In addition, our studies with TSL and SSL soils met the criteria for development of benchmarks suitable for derivation of Eco-SSL values for terrestrial plants.

Analysis of the results of the present studies showed that TNT was more toxic than RDX to all three plant species across all soil types. These results comport with recent studies that have shown that compared with cyclic nitramines, nitroaromatic EMs are more toxic to terrestrial plants in natural soils (Rocheleau et al., 2005, 2006; Robidoux et al., 2003; Simini et al., 1995; Palazzo and Leggett, 1983, 1986; Peterson et al., 1996; Gong et al., 1999).

In the present studies, toxicity of TNT W-A in soils to alfalfa, perennial ryegrass, and J. millet was in the order of TSL = SSL = KCL = RCL > WCL, based on the EC_{50} and 95% CI values for SFM and SDM. Toxicity was significantly greater ($p \leq 0.05$) in TSL, SSL, KCL, and RCL soils than in WCL soil for all three species based on the EC_{50} and the 95% CI values for the SFM and SDM endpoints.

In previous studies, soil concentrations of TNT and related nitroaromatic compounds (NACs) that caused phytotoxicity varied with soil type and plant species. Cataldo et al. (1989) reported a 50% reduction in plant height (*Phaseolus vulgaris* (L.) [bean], *Triticum aestivum* (L.) [wheat], and *Bromus mollis* (L.) [blando broom grass]) in two soils (1.7 and 7.2% OM) at 60 mg kg^{-1}, including a 25% reduction in plant height for wheat and blando brome grass at 30 mg kg^{-1} and no effects at 10 mg kg^{-1}. Gong et al. (1999) identified decreased seedling emergence values and increased phytotoxicity to *Lepidium sativum* (L.) (cress), *Brassica rapa* Metzg. (turnip), *Avena sativa* (L.) (oat), and wheat seedlings in two German soils amended with TNT. In that study, SFM decreased differentially among species. The cress SFM was significantly ($p < 0.01$) reduced at 50 mg kg^{-1} TNT, whereas oat and wheat SFM values were reduced at 150 and 350 mg kg^{-1}, respectively. The authors noted that differences in toxicity may be attributed to soil type.

Rocheleau et al. (2006) investigated the effects of TNT; 1,3,5-trinitrobenzene (TNB); 2,4-DNT; and 2,6-dinitrotoluene (2,6-DNT) FA (after a 24 h moisture equilibration period) or W-A (for 13 weeks) in SSL soil. Test chemicals were extracted from soil with ACN using U.S. EPA Method 8330 (U.S. EPA, 2007). The authors showed that on the basis of EC_{20} values for shoot growth (SDM), dinitrotoluenes were more phytotoxic for all species in FA treatments, which ranged from 3 to 24 mg kg^{-1}, compared with values for TNB or TNT, which ranged from 43 to 62 mg kg^{-1}. Exposure of the three plant species to relatively low concentrations of the four compounds initially stimulated plant growth before the onset of inhibition at greater concentrations. Seedling emergence values for J. millet and perennial ryegrass exposed to TNT, TNB, or 2,6-DNT W-A in soil significantly increased ($p < 0.05$), but shoot growth of all three plant species exposed to each of the four EMs significantly decreased ($p < 0.05$) compared with plants exposed to FA soil. The authors hypothesized that the formation of certain metabolites of the parent EMs detected in that study, such as 2- and 4-amino-4,6-dinitrotoluene (2- and 4-ADNT) and 3,5-dinitroaniline, may have contributed to decreased shoot growth after weathering-and-aging of EMs in soil. Formation of these chemicals may have contributed to the toxicity expressed in the present studies.

In the present studies, soils from the TNT phytotoxicity tests were only analyzed for TNT; analysis for other EMs was beyond the scope of this work. Benchmarks produced in the present studies are more representative of EMs that weather-and-age naturally on testing and training ranges than are those derived from EMs FA into soils (i.e., within a few days of testing). Under field conditions in which plants were exposed to natural wetting and drying cycles, TNT transformation products formed as the result of biotic and abiotic TNT degradation, including 2-ADNT; 4-ADNT; 2,4-diaminotoluene; 2,6-diaminotoluene; and TNB (Ainsworth et al., 1993; McCormick et al., 1976; Fernando et al., 1990; Esteve-Núñez et al., 2001; Hawari et al., 2000). In addition, 2,4- and 2,6-DNT are common byproducts found in munitions as impurities resulting from TNT manufacturing (Major et al., 2002).

Others have hypothesized that the bioavailability of TNT and related NACs in soil is dependent upon the OM content (Achtnich et al., 1999; Anzhi et al., 1997; Eriksson and Skyllberg, 2001; Simpson, 2006; Thorn and Kennedy, 2002), or the clay content (Emery et al., 2001; Haderlein et al., 1996), or a combination of the two (Jaenig, 2006). The WCL soil, which had the greatest percentages of both OM (5.3%) and clay (28%) among the five soil types in the present studies, produced the least resulting toxicities for all three plant species exposed. Results of Pearson's correlation analysis indicated that the soil clay content was significant ($p < 0.05$) and was the overriding soil property associated with decreased toxicity. From this we hypothesized that transformation and/or fixation of TNT was occurring in the soils tested. TNT, its metabolites, and other NACs have been shown to bond with the clay minerals (fixation) in soil (Daun et al., 1998; Esteve-Núñez et al., 2001). NACs react with the siloxane surface of clays to yield electron donor-acceptor complexes. In aqueous environments, adsorption of NACs to clays is high when the exchangeable cations at the clays include K^+ and NH_4^+ but is negligible for homoionic Na^+-, Ca^{2+}-, Mg^{2+}-, and Al^{3+}-clays (Haderlein et al., 1996; Weissmahr et al., 1997).

In the present studies, the effects of RDX on SFM and SDM were variable with respect to plant species and soil type. Perennial ryegrass was the most-sensitive species to RDX in these studies and was followed by J. millet. The SFM and SDM values of alfalfa grown in control soils were not significantly different from those for alfalfa plants exposed to any level of RDX in TSL, SSL, KCL, RCL, or WCL soils. Seedling emergence values for alfalfa, J. millet, and perennial ryegrass were not negatively affected by soil RDX concentrations in any of the soils studied. Chlorosis (yellowing due to decreased chlorophyll) of the leaf margins was observed on all plants grown in all RDX-amended soils in all soil types. Plants grown in negative-control soils did not show these symptoms.

In several recent studies, cyclic nitramine compounds such as RDX; octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX); and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) have shown little or no phytotoxicity (Gong et al., 2004; Rocheleau et al., 2005; Robidoux et al., 2003). Among these nitramines, RDX and HMX have been shown to be highly mobile within the plant and to concentrate in leaf and flower tissue (Harvey et al., 1991; Simini et al., 1995; Winfield, 2001) despite their relatively low water solubilities (Major et al., 2002). Therefore, these nitramines may pose risks to human health due to potential food-chain transfers to higher trophic levels (Harvey et al., 1991; Cataldo et al., 1989; Schneider et al., 1995; Major et al., 2002). Robidoux et al. (2003) found no reduction in seedling emergence or biomass of lettuce (*Lactuca sativa* (L.)) and barley (*Hordeum vulgare* (L.)), respectively, at analytically determined soil concentrations up to and including 3320 ± 1019 and 1866 ± 438 mg kg^{-1} HMX in artificial soil (OECD, 1984) or forest soil (3.8% OM, pH 7.6), respectively. Conversely, Winfield (2001) reported uptake and phytotoxic responses in 13 of 15 plant species subjected to short-term (≤12 days) RDX exposure. Toxicity symptoms included curled or irregular leaf margins, fused leaves, change in number of leaves per node, underdeveloped roots, curled root tips, necrotic leaves, yellow spots, chlorosis, and the expression of anthocyanin pigments. In general, the dicotyledonous plants in their study were more sensitive to RDX toxicity than were the monocotyledonous plants, and sainfoin (*Onobrychis viciaefolia* Scop.) and sunflower (*Helianthus annuus* (L.)) were by far the most sensitive of the species tested. The authors concluded that these symptoms were caused by

developmental effects and were indicative of DNA-toxicant interactions. In long-term experiments (2, 4, or 6 weeks) with sunflower, these symptoms were identified in 100% of the plants exposed to 6, 50, or 100 mg kg^{-1} RDX after 4 weeks (Winfield et. al., 2004). Significant root and shoot growth effects were found in some instances; however, the results were inconsistent. For example, biomass was significantly reduced at 50 and 100 mg kg^{-1} after 4 weeks' exposure to RDX but not at 6 weeks. Therefore, Winfield et al. (2004) concluded that symptoms attributable to developmental effects were consistent indicators of phytotoxicity to RDX, but growth was not. Our results in this study are consistent with those results. We observed chlorosis on leaves from all plant species at all RDX treatment levels (except controls) and across all soil types tested, but growth effects were variable among the three species studied.

Bioavailability and potential toxicity of TNT, RDX, and related EMs in soil depend on highly complex physical and chemical properties and environmental processes. The physical and chemical properties of soil OM and clay, as well as the total amount of these soil components, play a role in EM bioavailability. The binding of an EM to soil OM depends on the chemical and physical properties of the EM; the type of OM; and the ratio of fulvic acids, humic acids, and humin. Binding of EMs to soil clay varies; for the following clay minerals, binding typically occurs in the order of montmorillonite > illite > kaolinite. Binding of soil OM onto clay also varies with soil type depending on the nature and relative concentrations of the different types of OM and clay minerals present in the soil. In addition, environmental soil conditions such as moisture level, pH, ionic strength, and temperature may affect bioavailability. To further complicate matters, results of research show that binding sites in both OM and clays are finite, and that anthropogenically produced and naturally occurring organic compounds compete for these sites (Haderlein et al., 1996; Eriksson and Skyllberg, 2001). Very little research has been performed with mixtures of EMs and the subsequent toxic effects, and virtually nothing is known about synergistic or additive effects of EMs in natural soil. Therefore, predicting the relative bioavailability and potential toxicity of EMs on the basis of soil properties is difficult and includes a high degree of uncertainty. More extensive analysis is required regarding soil chemical and physical properties under established controlled environmental conditions, using soil types with substantial ranges of properties, to conclusively determine the role of these properties in determining EM bioavailability and toxicity to plants.

5. CONCLUSIONS

On the basis of the EC_{50} values and 95% CIs for SFM and SDM values, the toxicity of the nitroaromatic explosive TNT W-A in soil to alfalfa, perennial ryegrass, and J. millet was in the order (from greatest to least toxicity, according to soil type) of TSL = SSL = KCL = RCL > WCL. The results of these studies did not reveal a consistently strong relationship between TNT toxicity and the investigated soil properties; however, toxicity to each of the three species was significantly less when the plants were grown in WCL soil, which had the highest percentages of OM and clay among the soils tested.

The nitramine explosive RDX W-A in soils produced symptoms in plants that were indicative of developmental toxicity. RDX exposure was moderately inhibitory to growth of J. millet and perennial ryegrass in all soils tested but did not affect growth of alfalfa in the

present studies. In all soils tested, RDX concentrations were not appreciably different from nominal target concentrations after the weathering-and-aging process. RDX had relatively low sorption potential in soil and was readily leached through the vadose zone, which presents the potential for groundwater contamination. There was a strong inverse relationship between the RDX EC_{50} for SDM of perennial ryegrass and the soil clay content; however, this relationship did not exist for alfalfa and J. millet. This does not necessarily mean that soil properties are unimportant in determining TNT and RDX bioavailability and potential phytotoxicity.

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ACRONYMS AND ABBREVIATIONS

ACN	acetonitrile
2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2-6-dinitrotoluene
ANOVA	analysis of variance
ATCLP	adapted toxicity characteristic leaching procedure
BDL	below detection limit
BERA	baseline ecological risk assessment
CAS	Chemical Abstracts Service
CI	confidence interval
CL-20	2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane
2,4-DNT	2,4-dintrotoluene
2,6-DNT	2,6-dinitrotoluene
EC	effective concentration
EC_{20}	concentration that produces 20% decrease in measurement endpoint
EC_{50}	concentration that produces 50% decrease in measurement endpoint
ECp	estimate of effective concentration for a specified percent effect
Eco-SSL	ecological soil screening level value
EM	energetic material
ERA	ecological risk assessment
FA	freshly amended
FLSD	Fisher's least-significant difference
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	high-performance liquid chromatography
KCL	Kirkland clay loam
K_{ow}	octanol–water partition coefficient
LOEC	lowest-observed-effect concentration
NAC	nitroaromatic compound
NOEC	no-observed-effect concentration
OM	organic matter
p	probability
PTFE	polytetrafluoroethylene
QRB	qualitative relative bioavailability
R^2	regression sum of squares divided by total sum of squares
RCL	Richfield clay loam
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
RH	relative humidity
SDM	shoot dry mass
SFM	shoot fresh mass
SE	standard error
SLERA	screening-level ecological risk assessment
SSL	Sassafras sandy loam
TCLP	toxicity characteristic leaching procedure
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene

TSL	Teller sandy loam
USDA	U.S. Department of Agriculture
U.S. EPA	U.S. Environmental Protection Agency
W-A	weathered-and-aged
WCL	Webster clay loam
WHC	water-holding capacity


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